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Cyclosporin A in the prevention and treatment of experimental autoimmune glomerulonephritis in the brown Norway rat

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SUMMARY

Experimental autoimmune glomerulonephritis (EAG) was induced in brown Norway (BN) rats by a single i.m. injection of collagenase-solubilized homologous glomerular basement membrane (GBM) in Freund's complete adjuvant. This model of anti-GBM disease is characterized by the development, over several weeks, of circulating and deposited anti-GBM antibodies, accompanied by albuminuria. We examined the effects of treatment with oral cyclosporin A (CsA) at different doses, starting at the time of immunization and during the course of the disease. Pretreatment with CsA 5 mg/kg daily produced a moderate reduction in circulating anti-GBM antibody levels, reduced deposition of antibody on the GBM and decreased albuminuria. Doses of 10 and 20 mg/kg CsA produced a marked reduction in circulating antibody, absence of detectable deposited antibody and virtual absence of albuminuria. Renal function remained normal in CsA-treated and control animals. When CsA treatment was introduced at 2 or 4 weeks after immunization, there were significant effects on the subsequent autoimmune response and albuminuria at 10 and 20 mg/kg daily. These studies demonstrate that CsA in conventional doses has a therapeutic effect in this model of anti-GBM disease, and suggest a role for T lymphocytes in the pathogenesis of EAG.

Keywords autoimmunity glomerulonephritis glomerular basement membrane cyclosporin A brown Norway rat

INTRODUCTION

Autoimmunity to the glomerular basement membrane (GBM) is an important cause of crescentic glomerulonephritis, and this may be accompanied by lung haemorrhage to produce Goodpasture's syndrome (Wilson & Dixon, 1973). The pathogenicity of anti-GBM antibodies has been demonstrated by passive transfer experiments (Lerner, Glassock & Dixon, 1967), and control of antibody levels by plasma exchange and immunosuppressive drugs is accompanied by clinical improvement (Peters *et al.*, 1982). However, such treatment is non-specific and has appreciable side effects. The development of more specific immunotherapy depends upon better understanding of the mechanisms of induction and regulation of anti-GBM antibody synthesis, which may be approached by the investigation of appropriate experimental models (Wraith *et al.*, 1989).

The induction of experimental anti-GBM disease by administration of GBM antigens was first described by Steblay (1962) in the sheep. Subsequently, several other mammalian species have been shown to develop a similar experimental autoimmune glomerulonephritis (EAG) when appropriately immunized (Steblay, 1969). This has generally depended upon the use of heterologous GBM antigens in Freund's complete adjuvant (FCA), although we (Pusey *et al.*, 1984) and others (Sado & Naito, 1987) have found that certain rat strains also respond to homologous GBM. In our model, brown Norway (BN) rats given a single injection of homologous GBM in FCA develop sustained anti-GBM antibody production accompanied by albuminuria; focal proliferative glomerulonephritis and variable lung haemorrhage may also be observed (Pusey *et al.*, 1989).

The aims of this study were to investigate the effects of cyclosporin A (CsA) on prevention and treatment of EAG in the BN rat, examining the autoimmune response and the resulting renal injury. Since CsA is known to act principally on T_H lymphocytes (Shevach, 1985), these results should contribute to our knowledge of the mechanisms of EAG as well as suggesting possible therapeutic approaches.

MATERIALS AND METHODS

Experimental model

Female BN rats, aged 16–20 weeks and weighing 180–200 g, were obtained from our breeding colony. All animals were

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housed in standard conditions and had free access to normal laboratory diet and water.

Experimental autoimmune glomerulonephritis was induced by a single i.m. injection of collagenase-solubilized homologous (Sprague–Dawley) GBM in FCA at a dose of 2 mg/kg. Antigenic material was prepared from normal rat kidneys as previously described (Bowman, Peters & Lockwood, 1983). Serial blood samples were taken by tail artery puncture under ether anaethesia, and 24-h urine specimens were obtained by placing animals in metabolic cages. Animals were killed at 6 weeks and kidneys were removed for histological and immunohistological studies.

CsA (Sandoz, Basel, Switzerland) dissolved in olive oil was administered by gavage to groups of five animals at doses of 5, 10 and 20 mg/kg per day (for 6 days in each week), starting at day −1 prior to immunization and at days 14 or 28 during the course of the disease.

Control groups ($n=5$) were: (i) immunized and treated with olive oil; (ii) given CsA alone at the above doses; and (iii) given olive oil alone.

Assay systems

Anti-GBM antibodies were measured by a solid-phase radioimmunoassay, as previously described (Bowman *et al.*, 1983). Briefly, collagenase-digested BN GBM was coated onto microtitre plates (Dynatech Laboratories) by overnight incubation at 4°C, and test or control sera were applied for 1 h at 37°C. Binding of specific antibody was recognized by incubation with affinity-purified ^{125}I-labelled rabbit anti-rat IgG (Serotec) for 1 h at 37°C. Results were expressed as percentage binding of known positive pooled sera from $HgCl_2$-injected BN rats (Bowman *et al.*, 1983).

Urinary albumin concentrations were measured by rocket immunoelectrophoresis (Laurell, 1966) using rabbit antisera to rat albumin raised in our laboratory. Creatinine levels in blood and urine were measured by standard spectrophotometric methods. CsA absorption was assessed by measuring serum levels using the TDX fluorescent polarization immunoassay (Abbott Laboratories).

Renal histology

Direct immunofluorescence was performed as previously described (Cashman, Pusey & Evans, 1988) on kidneys post mortem 6 weeks after immunization. Tissue was embedded in OCT II embedding medium (BDH, Poole, UK) on cork disks, snap-frozen in isopentane (BDH) pre-cooled in liquid nitrogen, and stored at −70°C. Cryostat sections were cut at 5 μm and incubated with FITC-labelled rabbit anti-rat IgG (Dakopatts). The degree of immunofluorescence was assessed blind by a single observer (C.D.P.) and graded from 0 to 3+.

Tissue for light microscopy was fixed in 10% neutral buffered formalin and processed by standard techniques; 3-μm sections were stained with haematoxylin and eosin and periodic acid–Schiff.

Statistical analysis

Differences between data were determined by the two-sample Student's *t*-test. Analysis of variance was used to confirm differences between the multiple data (shown in Figs 1 and 5).

RESULTS

Cyclosporin A administration prior to immunization

Anti-GBM antibody levels. All control rats injected with GBM in FCA produced detectable circulating anti-GBM antibody, levels of which rose until week 6. CsA had a dose-dependent effect on antibody production. A dose of 5 mg/kg daily reduced antibody concentrations to intermediate levels ($P<0.005$), whereas doses of 10 or 20 mg/kg daily reduced antibody concentrations to levels close to those in unimmunized controls ($P<0.001$) (Fig. 1). Control animals given CsA alone or olive oil alone did not develop detectable circulating antibody.

Urinary albumin. The dose–response of CsA on urinary albumin excretion at week 4 (time of peak excretion in immunized controls) is shown in Fig. 2a. CsA given at a dose of 5 mg/kg daily reduced albuminuria significantly ($P<0.01$), while doses of 10 or 20 mg/kg daily reduced albumin excretion to nearly undetectable levels ($P<0.001$). Control animals given CsA alone (at all doses) or olive oil alone did not develop albuminuria.

Renal function. Serum and urine creatinine concentrations and creatinine clearances showed no significant differences between CsA-treated experimental groups and immunized or unimmunized controls.

Cyclosporin A absorption. Serum concentrations of CsA rose consistently as the dose of CsA was increased (Fig. 3), achieving levels comparable to those used therapeutically in humans.

Direct immunofluorescence. Direct immunofluorescence of kidney tissue at 6 weeks revealed that control animals given GBM in FCA and olive oil generally showed strong (3+) linear fluorescence for IgG along the GBM. Animals treated with CsA at 5 mg/kg showed weak linear fluorescence, and those given CsA at 10 or 20 mg/kg had no detectable fluorescence. Control animals given CsA at 20 mg/kg alone or olive oil alone showed no antibody binding. Results are summarized in Table 1 and illustrated in Fig. 4.

Light microscopy. None of the animals in any group showed evidence of proliferative glomerular change, tubular atrophy or interstitial damage. In some animals treated with CsA a

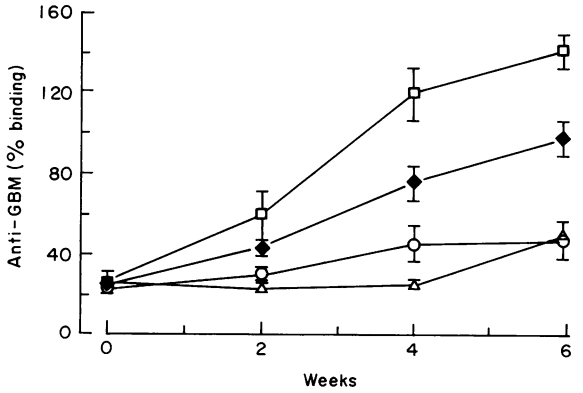

Fig. 1. Effect of cyclosporin A (CsA) given from day −1 on circulating anti-GBM antibodies in female BN rats ($n=5$) injected with Sprague–Dawley GBM in Freund's complete adjuvant. Results show mean ± s.d. for anti-GBM antibodies (% binding) in animals treated with oil (□); CsA 5 mg/kg per day (◆); CsA 10 mg/kg per day (○); and CsA 20 mg/kg per day (△).

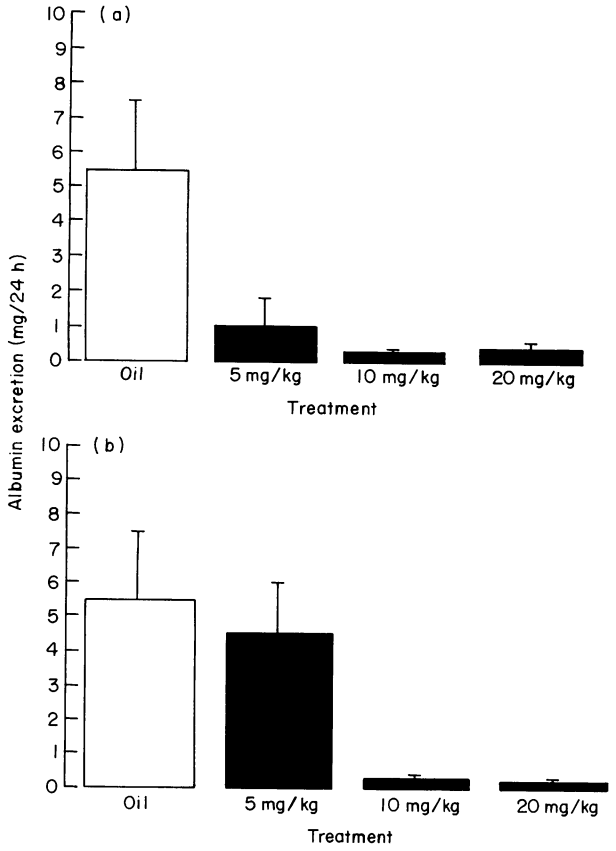

Fig. 2. Effect of CsA on albuminuria in female BN rats ($n=5$) injected with Sprague–Dawley GBM in Freund's complete adjuvant. Results show mean ± s.d. for albumin excretion in animals given CsA daily from (a) day −1 and (b) day 14.

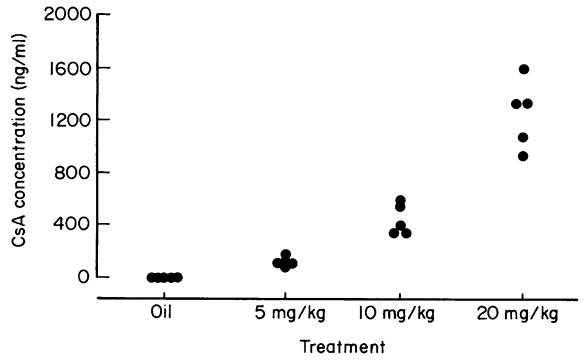

Fig. 3. Serum concentrations of CsA at week 4 in female BN rats ($n=5$) treated daily with different doses of CsA from day −1.

prominence of the juxtaglomerular apparatus was observed, but other kidney structures including vessels appeared normal.

Cyclosporin A administration during the disease

Anti-GBM antibody. When CsA treatment was delayed until week 2 following immunization, there was a dose-dependent effect on circulating anti-GBM antibody levels (Fig. 5a). When given at a dose of 5 mg/kg daily, there was no reduction in antibody levels at 4 or 6 weeks compared with immunized controls. However, when CsA was administered at 10 or 20 mg/kg daily, anti-GBM antibody levels were reduced at both week 4 ($P<0.005$) and week 6 ($P<0.005$), but were still higher than in unimmunized controls ($P<0.01$). Similar effects were seen when CsA administration was delayed until week 4, in that there was a significant reduction in antibody levels at week 6 in groups given CsA at 10 or 20 mg/kg ($P<0.01$) as compared with immunized controls (Fig. 5b).

Table 1. Effect of cyclosporin A given daily from day −1, day 14 and day 28 on deposition of IgG on the glomerular basement membrane (GBM), as judged by intensity of immunofluorescence, in female BN rats ($n=5$) injected with Sprague–Dawley GBM in Freund's complete adjuvant

	3+	2+	1+	−
Day −1				
Oil	5	0	0	0
5 mg/kg	0	0	4	1
10 mg/kg	0	0	1	4
20 mg/kg	0	0	0	5
Oil alone	0	0	0	5
20 mg/kg alone	0	0	0	5
Day14				
5 mg/kg	3	1	1	0
10 mg/kg	0	2	3	0
20 mg/kg	0	3	2	0
Day 28				
5 mg/kg	4	1	0	0
10 mg/kg	0	4	1	0
20 mg/kg	0	5	0	0

Urinary albumin. Urinary albumin excretion at week 4 in rats given CsA 10 or 20 mg/kg daily from day 14 was reduced to nearly undetectable levels as compared with immunized controls ($P<0.001$). Delayed treatment with CsA at 5 mg/kg daily did not reduce albuminuria (Fig. 2b).

Direct immunofluorescence. Renal tissue at week 6 from animals treated with CsA 10 or 20 mg/kg daily (starting at day 14 or day 28) showed moderate to weak linear fluorescence. Animals treated with 5 mg/kg daily showed strong fluorescence similar to that in immunized controls. Results are summarized in Table 1.

Light microscopy. No significant changes were seen.

DISCUSSION

This experimental model of Goodpasture's syndrome was developed in order to allow investigation of the induction and regulation of autoimmunity to the GBM. Studies in the rat are more practical than in larger species reported to develop EAG (Steblay, 1969), and our model in the BN strain avoids the use of heterologous antigens or polyclonal activators (Pusey *et al.*, 1991). The immunological characteristics of this model are more similar to the human disease than are those of the well-described $HgCl_2$-induced nephritis in BN rats, in which polyclonal activation occurs (Hirsch *et al.*, 1982; Pusey *et al.*, 1990). In

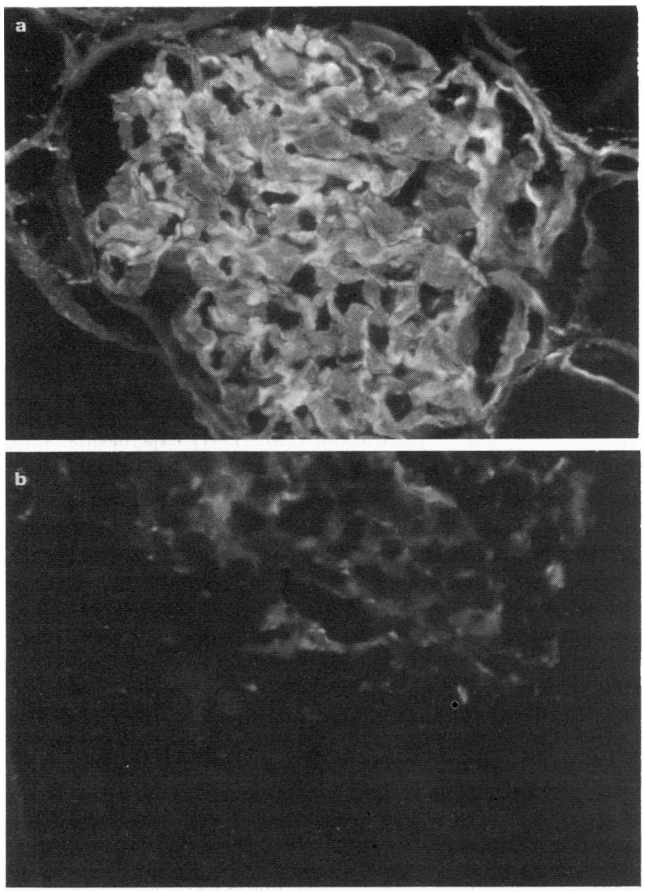

Fig. 4. Direct immunofluorescence of kidney tissue at 6 weeks from BN rats immunized with Sprague–Dawley GBM in Freund's complete adjuvant: (a) linear deposition of IgG along the GBM in an animal treated with oil; (b) negative findings in an animal treated with CsA 10 mg/kg per day from day −1.

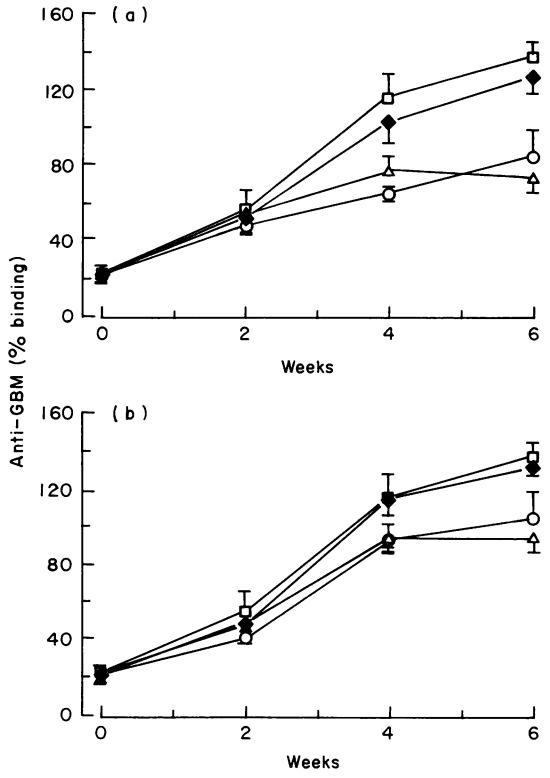

Fig. 5. Effect of CsA given from (a) day 14 and (b) day 28 on circulating anti-GBM antibodies in female BN rats ($n = 5$) injected with Sprague–Dawley GBM in Freund's complete adjuvant. Results show mean ± s.d. for anti-GBM antibodies (% binding) in animals treated with oil (□); CsA 5 mg/kg per day (◆); CsA 10 mg/kg per day (○); and CsA 20 mg/kg per day (△).

previous studies we have observed proteinuria and variable focal proliferative glomerulonephritis (Pusey *et al.*, 1991). However, in this series of experiments, albuminuria was not accompanied by extracapillary proliferation in glomeruli. This may be related to differences in the GBM digest used (although it was prepared by the same technique) or to differences between the BN rats concerned.

We chose to study the effects of CsA because of its principal mechanism of action, which is to inhibit IL-2 synthesis and release by T_H lymphocytes (Shevach, 1985; Hess & Colambani, 1986). Previous work has demonstrated the effectiveness of CsA in the prevention of various forms of autoimmune renal disease in the rat, including $HgCl_2$-induced nephritis (Aten *et al.*, 1988), Heymann's nephritis (Grönhagen-Riska *et al.*, 1990) and tubulo-interstitial nephritis (Giménez *et al.*, 1987). It is also effective in experimental diabetes mellitus (Laupacis *et al.*, 1983) and autoimmune encephalomyelitis (Feurer, Chow & Borel, 1988). However, in the telescoped model of nephrotoxic nephritis, treatment with CsA has been reported to cause both improvement (Tipping, Neal & Holdsworth, 1985) and worsening (Wood *et al.*, 1988) of glomerular injury.

The present study shows that CsA, in doses comparable to those used clinically, can inhibit the development of EAG. The effect appeared to be maximal at a dose of 10 mg/kg per day, and in the doses used there was no evidence of nephrotoxicity. In particular, reduction in albuminuria was not due to a reduced glomerular filtration rate. We did not observe increased morbidity or mortality at the higher doses, as reported by Baran *et al.* (1986). In general, there was concordance between the effects of CsA on autoantibody production and on proteinuria. Although circulating anti-GBM antibody was still detectable at the higher doses (10 and 20 mg/kg per day) of CsA, there was no detectable deposition of antibody on the GBM and virtually no albuminuria. At 5 mg/kg per day of CsA, circulating antibody levels were higher and weak antibody binding to GBM was detectable, as was slight albuminuria. The pathogenicity of autoantibodies in EAG in the rat has been demonstrated by Sado, Naito & Okigaki (1989), and our results support this finding. However, it remains possible that other mechanisms susceptible to CsA treatment, such as T cell-mediated injury, are also important.

Treatment of established nephritis with CsA (at the higher doses) was effective in reducing anti-GBM antibody levels and albuminuria, implying a continuing role for T_H cells in EAG. Similar effects of CsA in the treatment of established disease, rather than in prophylaxis, have been reported in tubulo-interstitial nephritis (Shih, Hines & Neilson, 1988) and $HgCl_2$-induced nephritis (Baran *et al.*, 1986) in the BN rat. However, delayed treatment was only effective until day 10 in one study of tubulo-interstitial nephritis (Giménez *et al.*, 1987). These observations suggest that CsA may be of clinical benefit in glomerulo-

glomerulonephritis, where treatment of an ongoing immune response is required.

We have shown that CsA is effective in the prevention and treatment of EAG in the BN rat, implying the presence of a T cell-dependent autoimmune response. This model of Goodpasture's syndrome should therefore be suitable for the investigation of more specific forms of immune intervention, directed at the trimolecular complex of autoantigenic peptide, MHC molecule and T cell receptor (Wraith *et al.*, 1989).

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